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(54) Title: METHOD AND APPARATUS FOR SYNTHESIS OF ARRAYS OF DNA PROBES

(57) Abstract: A method is described for the construction of DNA microarrays by *in situ* synthesis in which the DNA probes in the array are constructed from a series of small DNA polymers 2 to 4 nucleotides in length. The location of attachment of the small polymers in the microarray is determined by light actuated chemical deprotection of locations to which the small polymers are to be added.

METHOD AND APPARATUS FOR SYNTHESIS OF ARRAYS OF DNA PROBES

CROSS REFERENCE TO RELATED APPLICATION

Not applicable.

FIELD OF THE INVENTION

This invention pertains generally to the field of biology and particularly to techniques and apparatus for the manufacture of arrays of polymers useful in the analysis and sequencing of DNA and related polymers.

BACKGROUND OF THE INVENTION

The analysis of the genetic code of living organisms is a focal point of research in modern biology. As the entire genomic sequence of different species becomes available to science, the inquiry naturally turns from bulk sequencing operations to studies to determine the answers to questions like: What genes are active in what tissues? What genetic differences between individuals are associated with particular states of disease, physical attributes or behaviors? How can techniques of genetic analysis of individual patients or pathogens contribute to our understanding of disease?

Tools for genetic analysis are being developed to provide mechanisms to answer questions like these. Among such useful tools are what has become known as a DNA microarray. A DNA microarray is an array of single DNA strands arranged on a substrate. The DNA strands are organized into groups on the substrate, the groups being called a cell or a feature. Ideally, all of the DNA strands in a cell are identical in DNA sequence, and each of the cells can have an independent set of DNA strands of a different sequence. It is a chemical trait

of DNA that when single stranded DNA molecules are in solutions together at moderate or low temperatures, DNA strands of complementary sequence will spontaneously hybridize together through the formation of hydrogen bonds to form double stranded DNA. Thus a DNA microarray can be used to analyze a sample of unknown DNA (which has been made single stranded) to determine if complementary sequences are present in the sample simply by washing the unknown DNA sample over a microarray, and looking for the presence of double stranded DNA. DNA from the sample will hybridize to the array only when the sequence of the DNA from the sample matches the sequence in that particular cell. Thus, by intelligently designing and constructing DNA microarrays, which can contain thousands of these cells in a single array, it becomes possible to rapidly gather large amounts of information about the nucleic acids (DNA or RNA) contained in a sample in a simple and quick process.

Microarrays of DNA can be used for DNA sequencing, for the analysis of DNA from tissues samples to identify individuals or to diagnose disease conditions and for the analysis of mRNA levels in cells or tissues to study the tissue specific expression of native genes in any host.

To make a microarray of DNA strands, one can make a series of DNA strands and then place them on a support, or one can build DNA strands *in situ*. Both techniques have been used. The first technique, sometimes referred to as spotting, is convenient for the rapid and convenient creation of novel or small quantity custom arrays since the capital cost of making the DNA strands is not large. The spotting strategy can, however, be limited in the density of the array that can be created due to the physical limitations of droplets of liquids containing DNA that can be deposited on a surface. The strategy of creating DNA strands *in situ* on a surface permits the manufacture of arrays in which each cell of the array is very small and in which there are very many cells in a single array. Depending on the technique by which the DNA strands in the array are synthesized, however, the costs of making customized arrays can be quite high.

One technique for making DNA arrays by *in situ* fabrication is to use light sensitive chemical protecting groups to block unwanted DNA synthesis and then to de-protect

areas or cells selectively for the addition of nucleotide to the DNA strands in that cell. The original technique used to selectively guide light to the cells for this purpose was to use photolithographic masks, similar to those used in the fabrication of semiconductor integrated circuits. Using this technique, separate photolithographic masks are required for each of the nucleotide bases occurring in DNA, and separate masks are needed for each level of the array. Thus this technique if used to make an array of DNA molecules each of which is *N* nucleotides long, requires 4 times *N* masks in order to make even one DNA array. While the use of such masks permits the efficient synthesis of DNA microarrays, the costs of making an original mask set for any given chip can make the manufacture of a customized microarray for a low-volume application prohibitively expensive.

Another technique has been described for the *in situ* synthesis of DNA microarrays, a technique which is adapted for the manufacturing of customized arrays. Published PCT patent application WO99/42813, the specification of which is hereby incorporated by reference, describes a method for making such arrays in which the light is selectively directed to the array being synthesized by a micromirror array under software control from a computer. Since the micromirror array is operated totally under software control, the making of complex and expensive photolithographic masks is avoided in its entirety. This maskless approach to microarray fabrication permits the construction of DNA arrays directly from computer-stored DNA sequence information without intervening fabrication of intermediate materials.

One limitation involved in the synthesis of DNA strands *in situ* in an array arises from concerns about fidelity and accuracy of array contents. When DNA strands are synthesized off the array and delivered into the array as completed probes, the probes can be purified to any desired level of purity and checked for quality control prior to delivery. When the DNA strands are built in place, errors created in strand fabrication stay in the array. Since any chemical process has some inherent error and inefficiency, there will inherently be some probe strands that have improper sequence or sequence deletions. The amount of error compounds with the

number of chemical bonding steps that takes place during the manufacturing process. For example, if each step of the addition of a single nucleotide to a DNA strand were 90% efficient, 90% of the strands in a given cell would be accurate, and have the desired DNA sequence, after one nucleotide was added. After adding two nucleotides, 81% of the strands would be accurate. After three nucleotides, 72.9% of the strands would be accurate. The percentage of accurately constructed strands continues to decline as the strands become longer. Mathematically, the percentage of accurately constructed strands becomes less that 50% after the strands become 7 nucleotides in length. This obviously becomes a limitation on how long the strands can be and still be effective as an assay tool. While the actual efficiency of the nucleotide addition reaction is higher than 90%, the principle still holds that a limitation on the length of the strand is the efficiency of the nucleotide addition reaction. Since longer strands offer inherently better sensitivity for some applications (longer sequence that must be matched in the sample), fidelity of strand fabrication can become a limitation on the technology of *in situ* array synthesis. The longer DNA strands offer more unique sequences to test against samples in order to differentiate different cells or difference stages of cell growth.

SUMMARY OF THE INVENTION

In accordance with the present invention, the synthesis of arrays of DNA probe sequences, polypeptides, and the like is carried out rapidly and efficiently using patterning processes. The process may be automated and computer controlled to allow the fabrication of a one or two-dimensional array of probes containing probe sequences customized to a particular investigation. No lithographic masks are required, thus eliminating the significant costs and time delays associated with the production of lithographic masks and avoiding time-consuming manipulation and alignment of multiple masks during the fabrication process of the probe arrays. The probes are constructed from small DNA polymers, 2 to 4 nucleotides in length.

In the present invention, a substrate with an active surface to which DNA synthesis linkers have been applied is used to support the probes that are to be fabricated. To

activate the surface of the substrate to provide the first level of bases, a two-dimensional light image is projected onto the substrate, illuminating those pixels in the array on the substrate surface which are to be activated to bind a first base. The light incident on the pixels in the array to which light is applied deprotects OH groups and makes them available for binding to bases. After this development step, a fluid containing the appropriate small DNA polymers is provided to the active surface of the substrate and the selected base binds to the exposed sites. The process is then repeated to bind a different small polymer to a different set of pixel locations, until all of the elements of the two-dimensional array on the substrate surface have an appropriate small DNA polymer bound thereto. The small polymers bound on the substrate are protected with a chemical bound to the small polymers, and a new array pattern is then projected onto the substrate to activate the protecting material in those pixels to which the first new small polymer is to be added. These pixels are then exposed and a solution containing the selected small polymer is applied to the array so that the small polymer binds at the exposed pixel locations. This process is then repeated for all of the other pixel locations in the second level of small polymers. The process as described may then be repeated for each desired level of small polymers until the entire selected two-dimensional array of probe sequences has been completed.

Further objects, features and advantages of the invention will be apparent from the following detailed description when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings:

Fig. 1 is a schematic illustration of the methodology of the present invention.

Fig. 2 is a schematic view of an array synthesizer apparatus in accordance with the present invention.

Fig. 3 is a schematic view of another embodiment of an array synthesizer in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

In all of the prior work on DNA microarrays, the DNA probe strands were put in place in the array in one of two ways: either complete DNA strands were fabricated elsewhere and delivered to desired spot on the substrate of the array, or the DNA strands of the array were fabricated, nucleotide by nucleotide, *in situ* in desired location on the array. Here, an intermediate strategy is adopted. The DNA probes in the array are constructed from small polymers, containing two or more nucleotides, with the small polymers being selectively chemically bonded *in situ* to the building DNA strands in the array. By using small polymers in place of single nucleotides, the complexity of the instrument to make the arrays increases, but the increase in complexity can be managed. At the same time, the efficiency of array construction can actually be increased. Since one limitation on the efficiency of array synthesis arises from the number of chemical reaction which must be conducted to make a DNA probe, the length of the DNA probes which can be made for a given level of efficiency is increased in proportion to the number of nucleotides in the small polymers used. For example, adding nucleotide dimers to the building microarray, rather than single nucleotides, immediately doubles the length of the DNA strands which can be made *in situ* for a given level of efficiency, since DNA strands of twice the length can be made with the same number of chemical reactions.

One complexity of this approach arises from the number of reagents needed for the building blocks of the DNA strands. If it is decided to make the DNA array with dimers, in place of single nucleotides, the number of needed reagents for each synthesis step goes up by four times over those needed when using single nucleotides. When using single nucleotides, four reagents for addition to the DNA arrays are needed, for the four nucleotides represented by the letters A, T, C, and G. When dimers are used, 4^2, or 16, different reagents are needed, one for each combination of single nucleotides (i.e. AA, AT, AC, AG, TA, TT, etc.). If three base pair polymers are used, then 4^3, or 64, reagents are needed. For any given length n of polymer used in the process, the number of reagents needed is 4^n. Obviously, instrument complexity can rise with increasing efficiency, but at least for lower numbers of n, the complexity is manageable.

In addition, the length of time needed to make the DNA strands in the microarray may increase somewhat. For each step of adding onto the DNA strands in progress, instead of the four separate chemical reactions needed with a single nucleotide approach, for a dimer strategy there must be sixteen separate reactions, an increase of four times. However, since the process adds two base pairs to the growing DNA strands rather than one, the net increase of the time consumed by the actual chemical reactions increases only about two fold.

Note that it is the use of the digitally controlled light gate for the direction of light to the building microarray that makes this approach to DNA strand synthesis practical at all. If a mask-based photolithographic technique were used, and nucleotide dimers were used for DNA strand fabrication, the number of photolithographic masks needed for each step would go up by a factor of four. Since the costs of the design and making of these masks are a significant part of the cost of microarray synthesis by this approach, this incremental increase in costs might be difficult to accommodate. By contrast, in a micromirror based instrument, or any other instrument based on digitally controlled light gating, the costs of microarray synthesis do not increase substantially by virtue of using small polymers instead of single nucleotides to build a DNA microarray. The only additional inherent cost increase arise from the use of small polymers in place of single nucleotides is that the number of supply reagents needs to be increased to accommodate small polymers of all possible nucleotide sequences.

The general concept of making an array in this manner is illustrated in Fig. 1. In Fig. 1A, the entire surface of the substrate on which the array is to be made is covered with photolabile protecting groups ("P") by a linker ("O"). While any suitable photolabile protective groups can be used, the preferred chemistry uses 5'-[2-(2-nitrophenyl)-propyloxycarbonyl]-2'-deoxynucleoside phosphoramidites (NPPOC), as described in Hasan *et al., Tetrahdron*, 53:12, pp.4247-4264 (1997) and Beier and Hoheisel, *Nucl. Acids Res.* 2000, 28:4 (2000). As an alternative, the substrate can also be covered with a single nucleotide, or identical short polynucleotides, again with a photolabile protective group at their termini. The micromirror array is then illuminated to degrade the NPPOC in a selected array segment or cell where DNA

is to be added. This is illustrated in Fig. 1B. Then DNA dimers, in this case dimers of sequence AT, are exposed to the substrate, thereby chemically bonding to the array only in the cell to which the light had been directed by the micromirror array. This is illustrated in Fig. 1C. The small DNA polymers include another photolabile protective group appended to their terminus. Then this same process of light illumination and dimer addition is repeated for the dimer sequence AC, as shown in Fig 1D. This same process is then repeated 14 more times for each of the other possible DNA dimers that can be made from combinations of two nucleotides. At the end of the completion of a layer of the DNA probe synthesis process, as illustrated in Fig. 1E, two nucleotides have been added to each nascent probe in the microarray. This process is then restarted in the next level, and the process is repeated until the probes are built out to a desired length.

To illustrate the mechanisms for practicing the present invention, an exemplary apparatus that may be used for DNA probe array synthesis, using a micromirror light array, is shown generally at 10 in Fig. 2. This category of device is also illustrated in the disclosure of published PCT application WO 99/42813, the disclosure of which is hereby incorporated by reference. The apparatus includes a two-dimensional array image former 11 and a substrate 12 onto which the array image is projected by the image former 11. For the configuration shown in Fig. 2, the substrate has an exposed entrance surface 14 and an opposite active surface 15 on which a two-dimensional array of nucleotide sequence probes 16 are to be fabricated. For purposes of illustration, the substrate 12 is shown in the figure with a flow cell enclosure 18 mounted to the substrate 12 enclosing a volume 19 into which reagents can be provided through an input port 20 and an output port 21. However, the substrate 12 may be utilized in the present system with the active surface 15 of the substrate facing the image former 11 and enclosed within a reaction chamber flow cell with a transparent window to allow light to be projected onto the active surface. The invention may also use an opaque or porous substrate. The reagents may be provided to the ports 20 and 21 from a conventional DNA oligonucleotide synthesizer (not shown in Fig. 2).

/

The image former 11 includes a light source 25 (e.g., an ultraviolet or near ultraviolet source such as a mercury arc lamp), an optional filter 26 to receive the output beam 27 from the source 25 and selectively pass only the desired wavelengths (e.g., the 365 nm Hg line), and a condenser lens 28 for forming a collimated beam 30. Other devices for filtering or monochromating the source light, e.g., diffraction gratings, dichroic mirrors, and prisms, may also be used rather than a transmission filter, and are generically referred to as "filters" herein. The beam 30 is projected onto a beam splitter 32 which reflects a portion of the beam 30 into a beam 33 which is projected onto a two-dimensional micromirror array device 35. The micromirror array device 35 has a two-dimensional array of individual micromirrors 36 which are each responsive to control signals supplied to the array device 35 to tilt in one of at least two directions. Control signals are provided from a computer controller 38 on control lines 39 to the micromirror array device 35. The micromirrors 36 are constructed so that in a first position of the mirrors the portion of the incoming beam of light 33 that strikes an individual micromirror 36 is deflected in a direction oblique to the incoming beam 33, as indicated by the arrows 40. In a second position of the mirrors 36, the light from the beam 33 striking such mirrors in such second position is reflected back parallel to the beam 33, as indicated by the arrows 41. The light reflected from each of the mirrors 36 constitutes an individual beam 41. The multiple beams 41 are incident upon the beam splitter 32 and pass through the beam splitter with reduced intensity and are then incident upon projection optics 44 comprised of, e.g., lenses 45 and 46 and an adjustable iris 47. The projection optics 44 serve to form an image of the pattern of the micromirror array 35, as represented by the individual beams 41 (and the dark areas between these beams), on the active surface 15 of the substrate 12. The outgoing beams 41 are directed along a main optical axis of the image former 11 that extends between the micromirror device and the substrate. The substrate 12 in the configuration shown in Fig. 1 is transparent, e.g., formed of fused silica or soda lime glass or quartz, so that the light projected thereon, illustratively represented by the lines labeled 49, passes through the substrate 12 without substantial attenuation or diffusion.

A preferred micromirror array 35 is the Digital Micromirror Device (DMD) available commercially from Texas Instruments, Inc. These devices have arrays of micromirrors which are capable of forming patterned beams of light by electronically addressing the micromirrors in the arrays. Such DMD devices are typically used for video projection and are available in various array sizes, e.g., 640 x 800 micromirror elements (512,000 pixels), 640 x 480 (VGA; 307,200 pixels), 800 x 600 (SVGA; 480,000 pixels);1024 x 768 (786,432 pixels); and 1280 x 1024(SXGA; 1,310,720 pixels). Such arrays are discussed in the following article and patents: Larry J. Hornbeck, "Digital Light Processing and MEMs: Reflecting the Digital Display Needs of the Networked Society," SPIE/EOS European Symposium on Lasers, Optics, and Vision for Productivity and Manufacturing 1, Besancon, France, June 10-14, 1996; and U.S. Patents 5,096,279, 5,535,047, 5,583,688 and 5,600,383. The micromirrors 36 of such devices are capable of reflecting the light of normal usable wavelengths, including ultraviolet and near ultraviolet light, in an efficient manner without damage to the mirrors themselves. The window of the enclosure for the micromirror array preferably has anti-reflective coatings thereon optimized for the wavelengths of light being used. Utilizing commercially available 600 x 800 arrays of micromirrors, encoding 480,000 pixels, with typical micromirror device dimensions of 16 microns per mirror side and a pitch in the array of 17 microns, provides total micromirror array dimensions of 13,600 microns by 10,200 microns. By using a reduction factor of 5 through the optics system 44, a typical and readily achievable value for a lithographic lens, the dimensions of the image projected onto the substrate 12 are thus about 2,220 microns by 2040 microns, with a resolution of about 2 microns. Larger images can be exposed on the substrate 12 by utilizing multiple side-by-side exposures (by either stepping the flow cell 18 or the image projector 11), or by using a larger micromirror array. It is also possible to do one-to-one imaging without reduction as well as enlargement of the image on the substrate, if desired.

The projection optics 44 may be of standard design, since the images to be formed are relatively large and well away from the diffraction limit. The lenses 45 and 46 focus the light in the beam 41 passed through the adjustable iris 47 onto the active surface of the substrate. The

projection optics 44 and the beam splitter 32 are arranged so that the light deflected by the micromirror array away from the main optical axis (the central axis of the projection optics 44 to which the beams 41 are parallel), illustrated by the beams labeled 40 fall outside the entrance pupil of the projection optics 44). The iris 47 is used to control the effective numerical aperture and to ensure that unwanted light (particularly the off-axis beams 40) are not transmitted to the substrate.

The micromirror array device 35 may be formed with a single line of micromirrors (e.g., with 2,000 mirror elements in one line) which is stepped in a scanning system. In this manner the height of the image is fixed by the length of the line of the micromirror array but the width of the image that may be projected onto the substrate 12 is essentially unlimited. By moving the stage 18 which carries the substrate 12, the, mirrors can be cycled at each indexed position of the substrate to define the image pattern at each new line that is imaged onto the substrate active surface.

A variant of an array synthesizer apparatus, which uses an off-axis projection arrangement, is shown in Fig. 3. In the apparatus of Fig. 3, the light source 25 is provided with power from a power supply 50. The filter system 26 is composed is used to absorb infrared light and to selectively reflect light. A condenser 28, and with two plano-convex lenses 52 forms a Kohler illumination system. This illumination system produces a roughly collimated uniform beam of light with a diameter just large enough to encompass the active area of the micromirror array device 35. When the micromirrors are in a first position, the light in the beam 30 is deflected downwardly and out of the system. For example, in this micromirror device the mirrors in their first position may be at an angle of -10' with respect to the normal to the plane of the micromirrors to reflect the light well away from the optical axis. When a micromirror is controlled to be deflected in a second position, e.g., at an angle of + 10' with respect to the normal to the plane of the micromirrors, the light reflected from such micromirrors in the second position emerges perpendicularly to the plane of the micromirror array in the beam 41. The pattern formed by the light reflected from the micromirrors in their second position is then

imaged onto the active surface 15 of a glass substrate 12 enclosed in a flow cell 18 using a telecentric imaging system composed of two doublet lenses 45 and 46 and an adjustable aperture 47. As illustrated in Fig. 3, a standard DNA synthesizer 55 supplied with the requisite chemicals can be connected by the tubes 20 and 21 to the flow cell 18 to provide the desired sequence of chemicals, under control of the computer 38.

One variation necessary for the instrument of the present invention, as distinct from the instrument of the previously mentioned WO 99/42813 is that more chemical stocks are needed for the process. If nucleotides are added to the microarray one base at a time, then only four stocks are needed of DNA units, one stock for each of the four nucleotides in DNA. If the DNA units are added to the microarray in dimers, then the instrument needs to accommodate 16 stock solutions, one each for each of the possible two nucleotide dimers. This can be done most easily in one of two ways. One way is simply to use a DNA synthesizer 55 which can accommodate sixteen stock solutions, as some on the current commercial market today can do. The other alternative is to use more than one DNA synthesizer, each connected to fluid switches in the tubes 21 and 20 which are capable of switching the fluid connection to the flow cell 18 between the two DNA synthesizers. The fluid switches are then controlled by the computer 38 to switch between the synthesizer in coordination with the computer control of the synthesizers themselves to coordinate which synthesizer is supplying which stock solution at which desired time. This same arrangement can be used for more complicated supply designs when longer small polymers are used. If, for example, it is desired to make a highly accurate long strand probe microarray, DNA 4-mers could be used in the reaction, but then 256 stock solutions are needed. This would require a slightly more elaborate arrangement of tubing and a number of DNA synthesizers, but the principle would remain unchanged. The computer would control which synthesizer supplied which 4-mer at which step of the array synthesis process.

While described in particular with the synthesis of DNA arrays, it is to be understood that this same process and the same instrument can be used to fabricate any polymer by light activated chemistry including specifically other biomolecules such as RNA or proteins.

It is understood that the invention is not confined to the particular embodiments set forth herein as illustrative, but embraces all such modified forms thereof as come within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of making an array of DNA strands of selected sequence comprising the steps of

providing an instrument for making DNA array including a reaction chamber in which DNA synthesis reactions can be performed in a plurality of cells, the reactions using light labile chemical protectant groups so that light can be used to deprotect reaction products in a cell, a light source, an array of optical elements place in the optical path conducting light from the light source to the reaction chamber, and a computer operated by computer software, the optical elements of the array connected to be operable by the computer software;

placing in the reaction chamber DNA strands having light labile protective groups at their terminus;

selectively directing light to each cell under the control of the computer software by individually controlling the optical elements in the array to deprotect the DNA strands in selected cells; and

adding a small DNA polymer to the cells with deprotected DNA strands, so that the small DNA polymers are added to the DNA strands synthesized only in those cells selected by the computer software, the small DNA polymers consisting of 2 to 4 nucleotides.

2. The method of Claim 1 wherein the array of optical elements is selected from the group consisting of micromirrors, microshutters, and LCD crystal light shutters.

3. The method of claim 1 wherein the light labile protectant group is 5'-[2-(2-

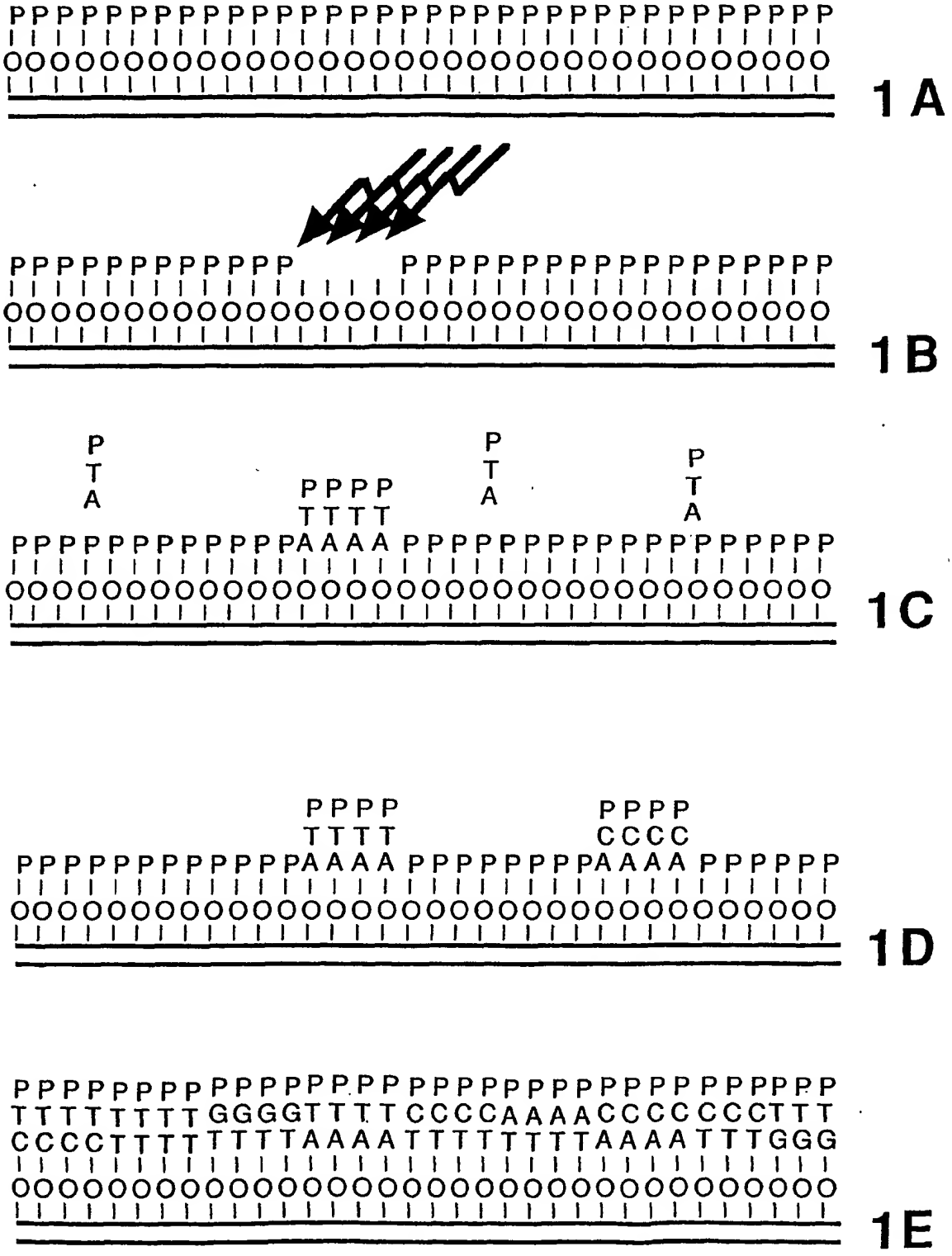
nitrophenyl)-propyloxycarbonyl]-2'-deoxynucleoside phosphoramidites (NPPOC).

4. The method of claim 1 wherein the small DNA polymers are dimers.

5. The method of claim 1 wherein the instrument includes a DNA synthesizer which supplies the DNA polymers to the process through tubing connected to the reaction chamber.

6. The method as claimed in claim 5 wherein there is more than one DNA synthesizer and the tubing includes switching operated by the computer to control which DNA synthesizer is feeding the DNA polymers to the reaction chamber.

FIG. 1



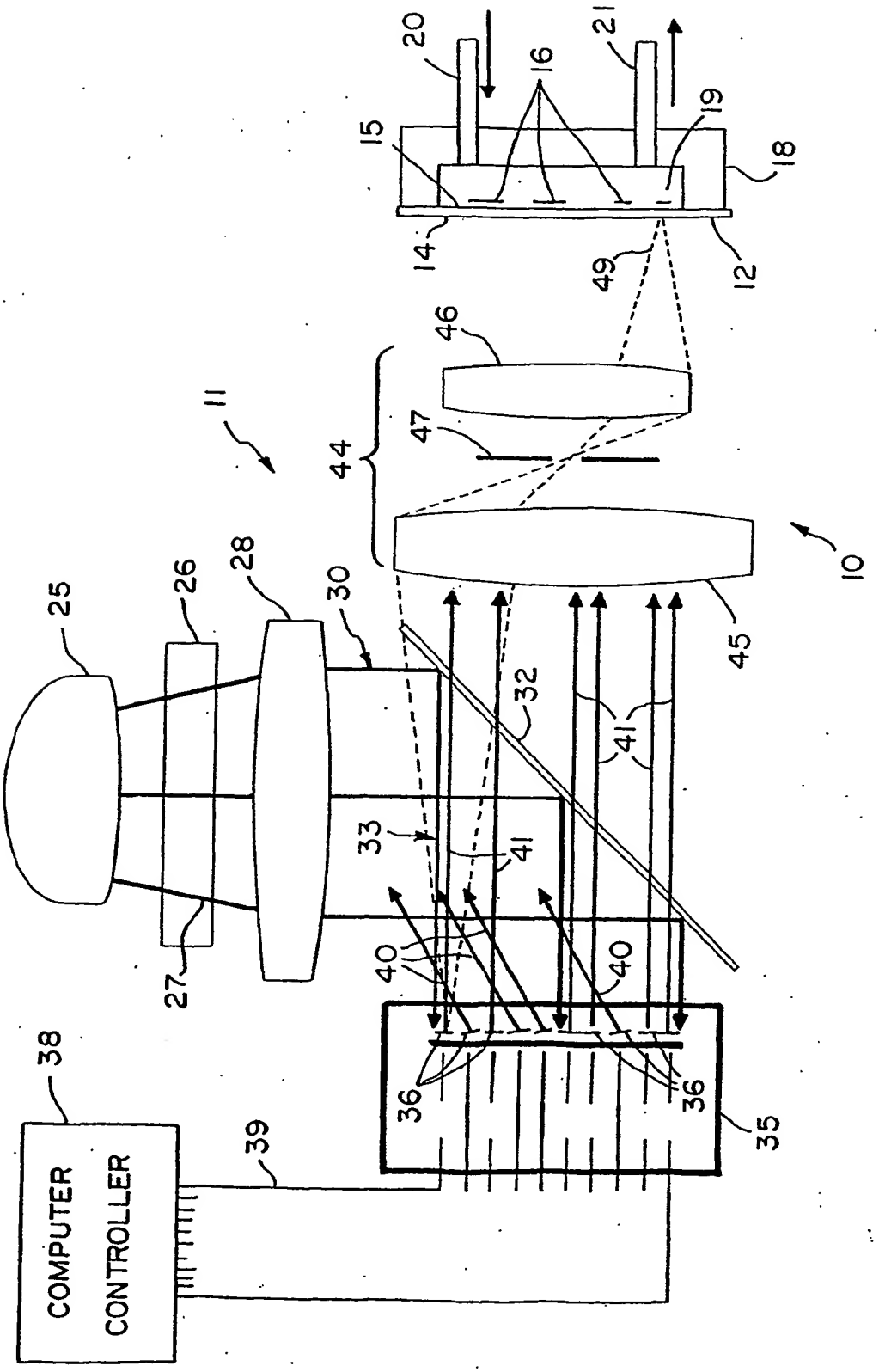

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FIG 2

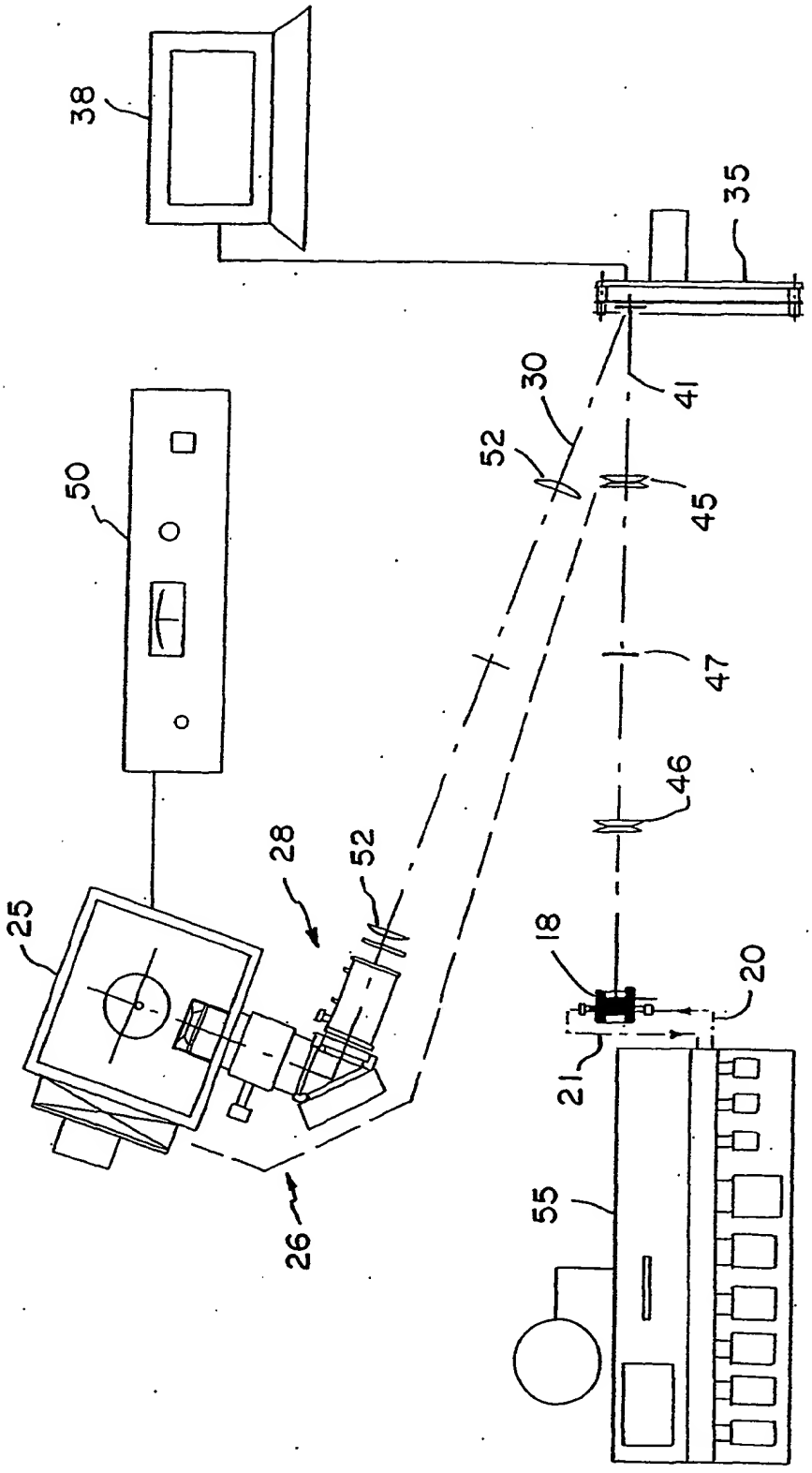

FIG 3